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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Berry et al.

Serial No.: 09/737,297

Filed: December 15, 2000

For: PROCESSES AND ORGANISM FOR THE PRODUCTION OF ANTI-FREEZE PROTEINS AND ANTI-FREEZE PROTEIN OBTAINED

Edgewater, New Jersey 07020
April 5, 2001

SUBMISSION OF PRIORITY DOCUMENT

Assistant Commissioner for Patents
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Sir:

Pursuant to rule 55(b) of the Rules of Practice in Patent Cases, Applicant(s) is/are submitting herewith a certified copy of the United Kingdom Application No. 9929696.4 filed December 15, 1999, upon which the claim for priority under 35 U.S.C. § 119 was made in the United States.

It is respectfully requested that the priority document be made part of the file history.

Respectfully submitted,

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Request for grant of a patent

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2.	Patent application number *(The Patent Office will fill in this part)*	**9929696.4** 15 DEC 1999
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	**UNILEVER PLC** **UNILEVER HOUSE, BLACKFRIARS** **LONDON, EC4P 4BQ**
	Patents ADP number *(if you know it)* 1628002	
	If the applicant is a corporate body, give the country/state of its incorporation	**UNITED KINGDOM**
4.	Title of the invention	**PROCESSES AND ORGANISMS FOR THE PRODUCTION OF ANTI-FREEZE PROTIENS**
5.	Name of your agent *(if you have one)*	**KIRSCH, Susan Edith**
	"Address for Service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**PATENT DEPARTMENT, UNILEVER PLC** **COLWORTH HOUSE, SHARNBROOK** **BEDFORD, MK44 1LQ**
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11. I/We request the grant of a patent on the basis of this application.

Signature(s) Date: **15-Dec-99**

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PROCESSES AND ORGANISMS FOR THE PRODUCTION OF ANTI-FREEZE PROTEINS

The present invention relates to a process for the production of antifreeze proteins. It further relates to novel organisms useful in the process, to novel proteins obtained thereby and to compositions and uses of such novel proteins. In one aspect, it relates to cultures of the novel organism *Marinomonas protea*; to novel antifreeze proteins derived therefrom; to the use of such proteins in controlling freezing processes, especially in the production of frozen food; and the foods thereby obtained.

So-called 'antifreeze proteins' (AFPs) have the property of modifying the growth of ice crystals. They differ in their action from simpler ionic antifreeze agents such as common salt. For example, aqueous AFP solutions typically have a freezing point that is lower than their melting point (hysteresis). They stabilise ice crystals over a range of temperatures, and inhibit recrystallisation. They seem to assist organisms to survive in temperatures around the freezing point of water, and are accordingly found in several different types of organism. Their properties give them a range of potential uses: in particular in foods that are eaten while frozen, by inhibiting recrystallisation and maintaining a smooth texture. In foods that are frozen only for preservation, AFPs may inhibit recrystallisation during freezing, storage, transport, and thawing, thus preserving food texture by reducing cellular damage and also minimising the loss of nutrients by reducing drip (see Griffith, M. and Vanya Ewart, K. *Biotechnology Advances*, 13, pp 375-402).

Various sources of AFPs are known: the commonest are fish and plants. Some bacteria exhibit antifreeze properties (see Griffith et al, supra, p 382): in a few cases, the isolation of antifreeze proteins from bacteria has been reported (see for example: Xu H, Griffith M, Patten CL, et al., Can J Microbiol 44: (1) 64-73 Jan 1998). Given the advantages to an organism of resistance to freezing, it may be considered surprising that anti-freeze proteins are not more widely distributed in Nature and easier to find. This may be because organisms have other ways of overcoming such problems. The hypothesis on which the present invention is (in part) based is that bacteria are more likely to evolve AFP proteins if they inhabit a liquid aqueous environment which is often below the normal freezing point of water: in such environments (it is postulated) AFPs may be an efficient way of giving bacteria a competitive advantage.

Accordingly, in a first aspect, the present invention consists of a process for producing AFPs which comprises collecting one or more samples of bacteria from an aqueous low-temperature environment, extracting proteins from the samples, testing the proteins for antifreeze properties, selecting protein having superior antifreeze properties, and producing the selected protein in amounts sufficient for use as an AFP food additive. The invention further comprises novel anti-freeze proteins obtainable by the process, their use in food processing, and food compositions containing them.

By an aqueous low-temperature environment, we mean an environment comprising predominantly water that is at a temperature below 0°C for at least part of the year. We prefer saline environments, for example having a salt content (salinity) sufficient to depress the freezing point of ice significantly (for example by 0.2°C or more). The salinity may be more or less than that of sea water (35ppt). Preferred environments from which bacteria can be collected are saline (including hypersaline) lakes, or loci in such lakes. In particular we prefer to collect suitable bacteria for use in the invention from Antarctic environments.

Saline lakes in Antarctica are often meromictic or monomictic. Meromictic lakes have a permanent chemically and thermally stratified water column. They have colder less saline waters above and more saline warmer waters in the depths. In Antarctica such lakes are ice-covered for most of the year, only breaking out briefly in summer. Monomictic lakes in Antarctica are hypersaline lakes, too saline to develop a proper ice-cover in winter. Ice-cover acts like a thermal blanket. Without it lake waters in contact with the air cool to very low temperatures. In winter their water columns stratify thermally, colder waters above, warmer waters below. In summer the stratification breaks down and the water column has a uniform temperature. In the case of both monomictic and meromictic Antarctic lakes there are times in winter when water temperatures are well below 0°C.

The invention further comprises pure bacterial cultures of the novel bacterial species *Marinomonas protea*. Such cultures are obtainable in the course of carrying out the process of the invention. The invention further comprises the novel AFP marinomonin which may be isolated therefrom: processes of preparation of marinomonin which comprise culturing an organism containing a gene coding for marinomonin under conditions in which marinomonin is produced, and recovering marinomonin; and food preparations containing marinomonin as a stabilising agent.

We further provide pure bacterial cultures of *Marinomonas* species that generate antifreeze proteins, said bacterial cultures showing at least 90% and preferably 95% homology in the 16S mRNA gene sequence with the corresponding sequence in the organism *Marinomonas protea* (Figure 3).

The process of the invention comprises four phases: collection of samples; extraction of proteins; testing and selection of proteins; and production of proteins.

1. Collection of samples

Samples of bacteria for use in the process of the invention are collected from aqueous low-temperature environments. As noted, by 'low temperature' environments we mean those in which ice forms for at least part of the year. Such environments occur at high altitudes and high latitudes, or both. We prefer to collect bacteria from saline environments, especially saltwater lakes. Particularly preferred environments are found in the Antarctic, and may include meromictic or monomictic lakes. Such lakes comprise

loci having a variety of properties, e.g, temperatures and salt concentrations, and bacterial samples may be collected from a range of such loci for subsequent laboratory investigation.

2. Protein extraction

Each bacterial sample is cultured under suitable conditions, and protein extracted from the culture broth by standard methods, for example by centrifugation of cells, pelleting and vortexing with glass beads in the presence of buffer.

3. Testing of proteins

Proteins extracted in stage 2 are classified by testing their anti-freeze properties. A variety of suitable tests are available. We prefer to use a recrystallisation inhibition test: as described in more detail below. This measures the tendency of ice crystals to increase in size when stored under freezing conditions in the presence of the protein. By use of this test, effective AFP proteins, in the presence of which recrystallisation and crystal growth is stopped or reduced, may be selected.

4. Production of proteins

Proteins selected according to the process of the invention are produced in quantity for use as AFP food additives. This may be done in a variety of ways. For example, the original process used to obtain the protein (bacterial culture followed by protein extraction) may be scaled up. By experimenting with culture conditions (temperature, media, etc.) and bacterial selection, yields may be increased. It is also possible to isolate from the

bacterium the DNA sequence coding for the selected AFP, introduce it into an expression cassette and generate recombinant protein in a different host cell.

The invention will be further described with reference to the accompanying drawings, in which:

Figure 1 shows growth curves of the organism *Marinomonas protea* in Tryptic soya broth (BS, made up with non-saline water);

Figure 2 shows growth curves of the organism *Marinomonas protea* in sea salt medium (of comparable salinity to the environment from which it was isolated);

Figure 3 is a DNA sequence from the 16S rRNA gene of *Marinomonas protea;*

Figure 4 shows the sequence alignment of the 16S rRNA sequence of *Marinomonas protea* (Figure 3) to the corresponding sequence of *Marinomonas communis;*

Figure 5 is a phylogenetic tree comparing *Marinomonas protea* with its closest phylogenetic relatives.

Example 1

19 Isolates were collected from various sites in Antarctic lakes. Two were found to give rise to anti-freeze proteins (see Table 1 below), isolate 196 and isolate 20. These bacteria were isolated from Ace lake and Club lake, respectively. Ace lake is meromictic: Club lake is monomictic. Both bacteria were isolated from near to the surface of the lake. Isolate 196 was isolated from the ice/water interface. Isolate 20 was isolated from the top

50cm of water column away from the edge of the lake. More details of the two lakes are provided below:

Ace lake

Brackish at surface - 18ppt; salinity increasing with depth to 34 ppt.

Ice cover for 10-11 months of the year

Meromictic - chemically and thermally stratified

Club lake

Hypersaline (250 ppt)

No ice cover

Monomictic - stratified thermally in winter, water column mixed in summer

Culturing of Isolates

Bacterial isolates were inoculated into 200ml of Tryptic Soya Broth (Oxoid).

Isolate 196 (*Marinomonas protea*) was also cultured in 200ml Sea Salts Broth and 200ml of ½ Sea Water Broth

Tryptic Soya Broth ["TSB"] (Oxoid):	Pancreatic digest of Caesin	11.3g/L
	Papaic Digest of Soya Bean	2g/L
	Dibasic Potassium phosphate	1.7g/L
	Glucose	1.7g/L
	NaCl	3.3g/L
	pH	7.3 +/- 0.2

Sea Salts Broth ["SSB"]:	Sea salts (Sigma)	40g/L
	Peptone (BDH)	5g/L
	Yeast Extract (Merck)	2g/L
Sea Water Broth ["SWB"]:	'Instant Ocean' (Aquarium Systems, France)	38g/L
	Yeast Extract (Merck)	1g/L
	Peptone (BDH)	1g/L
½ Sea Water Broth ["½SWB"]:	'Instant Ocean' (Aquarium Systems, France)	19g/L
	Yeast Extract (Merck)	1g/L
	Peptone (BDH)	1g/L

All cultures were incubated at 15°C until noticeably turbid (this took 2-6 days depending on the growth rate of each culture). All cultures were then cold shocked at 5°C for 4 days. 200mls of an *E. coli* culture was also produced as a negative control. This was strain W3110. It was cultured in Lauria-Bertani ["LB"] media in a shaking vessel at 37°C overnight.

LB media	Bacto-tryptone	10g/L
	Bacto-yeast extract	5g/L
	NaCl	10g/L
	Adjusted to pH 7.0	

Protein extraction

The following protocol gives the method used for total cellular protein extraction.

1. The cells were collected from the cultures by centrifugation. A Beckman cooling centrifuge, with a fixed angle JA-14 rotor was used. Samples were spun at a constant temperature of 4 °C and at a speed of 10,000 rpm (15,300g) for 10mins. The supernatant was carefully removed leaving the pellet.
2. The pellet was resuspended in 1ml of 10mM Tris/HCL buffer (pH 7.0). This was repelleted using the same centrifuge conditions as step 1. The supernatant was discarded carefully leaving just the pellet.
3. The pellet was then resuspended in 1ml of protein extraction buffer (see below) and then kept on ice for 5mins to allow cell lysis. The resuspended pellet was then sonicated in a thick walled bottle using a Sanyo Soniprep 150 at 15 A for 30 seconds.
4. The sonicated mixture was then transferred back to a centrifuge tube and centrifuged as outlined in step 1. The supernatant was removed and frozen at -20°C until required.

Protein Extraction Buffer: 25mM Tris/HCL (pH 7.0)
1mM EDTA
1mM PMSF (Sigma)
2ug/ml Pepstatin A (Sigma)

Splat Assay

The following protocol is the method used to view the level of ice re-crystallisation inhibition achieved by each of the protein extractions. It is a modification of the assay described in Byass et al. [Unilever WO 9804148].

1. A 60% sucrose solution was prepared. A 20μl sample of each protein extract was mixed with 20μl of the sucrose solution. These mixtures were made up in 1.5ml Eppendorf tubes. The 30% sucrose/protein extract solutions were spun in a MSE Micro Centaur Desk top micro-centrifuge for 10 seconds to make sure all the liquid was mixed at the bottom of the tube.

2. 5μl of the resulting solution was placed between two circular 16mm diameter coverslips upon which the number of the extract was written in waterproof maker. These were then blotted dry.

3. The coverslips were then dropped into 2,2,4 - Trimethyl pentane that had been pre-chilled to -70°C (using dry ice). They were super-cooled in this for 2 minutes.

4. A chilled bath was filled ¾ full of 2,2,4 - Trimethyl pentane, which was pre-chilled to -6° C using a Haake C water bath circulator and two Haake temperature control units (PG40 and F4).

5. The coverslips were removed from the super-cooling process and placed directly into the -6° C bath for 30mins to allow re-crystallisation.

6. The coverslips were then viewed whilst in the -6° C bath using a EF L 20/0.30 160/0-2 objective on a Leitz Dialux 20 EB stage.

7. The observed crystal shape, size and density were related to the level of AFP activity using the Splat Scoring System outlined below.

Splat Score	Observed crystal morphologies
+++++	Very small, very dense crystals.
++++	Small, not dense; or small, quite dense with some medium-sized crystals.
+++	Small, not dense with medium-large crystals; or small-medium, not dense.
++	Medium or large crystals with some small crystals
+	Large, round discrete crystals (e.g. 30% sucrose control).

[A score of +++ or above is required for an isolate to have any practical utility].

Results from the splat assay are shown in Table 1

TABLE 1

Bacterial isolate	**AFP activity**	**Bacterial isolate**	**AFP activity**
196 [1/2 SWB]	++++	196 [TSB]	+++
196 [SSB]	++++	18	++
20	+++/++++	26	+
29	+/++	39	+
43	++	44	+
98	++	101	+
104	+	118	++
120	+	125	+
184	+	226	+
288	+	121	+
289	+	*E. coli* (neg. control)	+
Sucrose only (neg. control)	+		

The results in Table 1 show that out of the 19 isolates of Antarctic bacteria that were investigated, only isolate 196 and isolate 20 produce proteins with significant amounts of AFP . Isolate 196 *is Marinomonas protea*, as further described below.

Example 2

Collection of Isolate 196

Bacteria were isolated from the ice/water interface of Ace Lake, Antarctica (68°28′S, 78°11′E) on 31/6/96. Samples were collected by drilling a hole through the ice (1600 mm thick) at the deepest point of the lake. Sections of the lower part of the core from the ice/water interface were melted onto agar. The material was designated Isolate 196.

Growth characteristics of Isolate 196

Colonies of isolate 196 that formed on half-strength seawater agar after 3 to 5 days of incubation at 10°C were non-pigmented (creamy colour), smooth, convex, circular with entire edges and ranged in size from less than 0.5 mm to 1.5 mm in diameter. On tryptic soy agar the colonies were beige in colour, mucoid and spreading, and ranged from 2-5 mm in diameter. The isolate was psychrotrophic, growing at temperatures between <0 to ~25°C (optimal growth between 15-22°C), with no growth at 30°C. The bacterium grew aerobically and under microaerophilic conditions, but would not grow anaerobically. It did not require seawater for growth, but could tolerate up to 70‰ NaCl. Cells growing in 60-70‰ NaCl consisted of straight rods and coccoid cells, which appeared more wrinkled and indented than cells in lower salinity media. Typical growth curves in different media types are shown in Figures 1 and 2.

Organism Classification

16S rRNA Phylogenetic Analysis Isolate 196

Identification of the isolate was based on 16S rRNA analysis. A near complete (1,485-bp) sequence of the 16S

rRNA gene, stretching from nucleotide positions 18 to 1503 (*Escherichia coli* equivalent numbering), was obtained and is shown in Figure 3. 16S rRNA analyses against species in the publicly available databanks showed that it represented a novel species of the genus *Marinomonas* (in the gamma-3 subclass of the phylum Proteobacteria), with 74.7% identity to *Marinomonas communis* (the type species of the genus).The match between the two sequences is shown in Figure 4. The name *Marinomonas protea* sp. nov. (*pro´te.a.* Gr. n. *Proteos,* name of the Greek mythological god of the sea who could appear in many shapes, M.L. fem. adj. *protea*, polymorphous) was proposed (Prof. Hans G. Trüper, *pers. comm.*). The bacterium has been deposited at the British National Collections of Industrial and Marine Bacteria, Aberdeen (NCIMB) under the number 41006 on 9 February 1999.

The 16S rRNA generated in this study were deposited in GenBank. Percentage similarities for the sequences are given in Table I. The phylogenetic tree determined using PHYLIP (version 3.57c) is shown in Figure 5 . It uses almost complete 16S rDNA sequences, comparing *Marinomonas protea* with its closest phylogenetic relatives and with three other sea-ice isolates identified as *Marinomonas* sp. (M. Brown, *pers. comm*). The tree was constructed using the maximum likelihood method (Felsenstein, 1981).

16S rRNA Phylogenetic Analysis of Isolate 20

A partial analysis of Isolate 20 found it to be a species of *Pseudomonas*. More precisely, isolate 20 was found to have a similarity value of 98.8% with *Pseudomonas azotoformans*.

Example 3

Total Cellular Protein Extraction

A bacterial culture (2 L) of Isolate 196 was grown in liquid medium (Tryptic Soy Broth) at 15°C until turbid. The culture was aseptically pipetted into sterile Nalgene™ universals. The cells were harvested by centrifugation at 4,200 x g for 12 minutes at 5°C (Sorvall Dupont Econospin). The pellets were resuspended in 1 ml of ice-cold 10 mM Tris buffer (Tris [hydroxymethyl]amino-methane)/HCl (pH 7.0) The cells were pelleted for 10 minutes. The pellets were then either stored at -20°C overnight, or protein extractions were carried out immediately. The pellet was resuspended in 1 ml of ice-cold native extraction buffer (NEB, which consisted of 25 mM Tris/HCl (pH 7.0), 1 mM EDTA, 1 mM PMSF and 0.1 mM Pepstatin A). The Tris buffer acts as a biochemical buffer, the EDTA is a chelating agent and is active against metalloproteases, PMSF is an inhibitor of serine proteases and Pepstatin A inhibits proteases. To each resuspended pellet, two volumes of acid- (70% HCl) washed glass beads were added (1.5 ml of 425-600 µm diameter beads and 0.5 ml of 3 mm diameter beads, both Sigma), or enough to create a viscous adherent coating of cell suspension over the beads. The beads were acid washed to prevent alkali being liberated from the glass when vortexed, as this would potentially damage alkali-sensitive components of the cells. The mixture was then vortexed for 1 minute sessions, with 1 minute intervals on ice. This was repeated 8 times for each sample, after which a further 1 ml of ice-cold NEB was added to the beads, which were re-vortexed to enable the cell suspension to go into solution. The cells were left on ice for 5

minutes to allow the glass beads to settle and the lysates were transferred to 1 ml sterile, ice-cold Eppendorf tubes and centrifuged (Beckman MSE, 12,000 x g, 5°C, 5 minutes) in order to separate the cell walls and non-ruptured cells, as well as any glass beads which were accidentally transferred. The supernatant was transferred to sterile, ice-cold Eppendorfs and centrifuged once more to remove any further cell debris that may interfere with the RI assay. The supernatant was pipetted into 2 ice-cold, labelled Eppendorfs and stored at -20°C until the assays were carried out.

Example 4

Effect of Bacterial Antifreeze proteins on Ice Crystal Morphology

The effect of the antifreeze protein from isolate 196 (obtained as in Example 3) on ice crystal morphology was assessed by examining total cellular protein extract at -6°C under the microscope. Hexagonal ice crystals, some elongated, were clearly visible.

Example 5

Protein Purification and Sequence Information

Purification of Active Protein

The bacterial antifreeze protein, which we term marinomonin, was isolated from the crude extract produced in Example 3 using reverse phase chromatography (RPC) on an AKTA™ protein purification system, followed by gel exclusion chromatography (Sephadex® 75, or S75) on a SMART™ system (both Pharmacia Biotech).. The active fractions from each

separation method were run on a 10% bis-acrylamide SDS gel: the band obtained ran at a molecular weight of ~38 kDa

Sequence Information

The 38 kDa protein band was electroblotted onto a PVDF membrane and N-terminal sequenced. The following sequence of 16 amino acids was obtained, with the 6th amino acid producing an ambiguous result of either Glycine or Valine (shaded region indicates region of sequence that the oligonucleotide probe was designed from):

Ala-Glu-Gly-Ser-Thr-Gly/Val-**Asp-Val-Tyr-Gln-Asn-Ile-Gln-Tyr-Ala-Gly**

Database Homology Searches

Sequence homology searches were carried out using the N-terminal sequence of the novel AFP protein using FASTA searches against the SWISS-PROT database, which included all published AFP sequences. The sequence matches were random, in that similarities were matched to a wide range of organisms, both bacterial and eukaryotic, and the similarities were not particularly high. Also, the regions of homology between the protein sequences were not located at the N-terminus of the proteins on the database, but mid-sequence: this reduces the likelihood of any real homology between the protein sequences and the N-terminal sequence of this novel protein.

As previously stated, the invention comprises the novel protein marinomonin having antifreeze properties. It further comprises isoforms and derivatives, e.g.

glycosylated forms, of marinomonin possessing antifreeze properties. Preferably the derivatives show at least 75%, more preferably 85% and in particular at least 95% homology with the 16-member peptide sequence given above.

The invention further comprises DNA sequences encoding the novel proteins of the invention. These may readily be obtained from a knowledge of the protein sequence shown above.

The process of the invention comprises culturing an organism containing a DNA sequence coding for marinomonin under conditions in which marinomonin is produced, and recovering marinomonin.

One such organism suitable for use in the invention is the newly discovered bacterium *Marinomonas protea*. Suitable conditions and media for culturing this organism are given above.

Other organisms may be discovered containing DNA encoding marinomonin, which may prove useful in the process of the invention. An alternative is to employ a genetically transformed organism comprising a DNA sequence coding for an anti-freeze protein which is marinomonin or an analogue thereof, the sequence being under control of a gene promoter adapted to cause it to produce the antifreeze protein in the transformed organism. The DNA sequence coding for the antifreeze protein may be inserted into a suitable expression vector containing the necessary elements for transcription and translation into the desired protein under

appropriate conditions, including proper orientation of the sequence, correct reading frame, and suitable targeting and expression sequences. Methods for making suitable vectors, and for using them to transform many different types of organism, are well understood in the art. Genetically transformed organisms suitable for carrying out the process of the invention also form a further aspect of the invention.

In principle, any organism may be modified to produce the desired protein in this way, for example, bacteria, yeasts, plants, or plant, insect or animal cells. Bacteria, yeasts and plants or plant cell systems are generally preferred.

It is also possible to provide suitable genetically transformed organisms, adapted to produce the AFPs of the invention, which are themselves useful on account of their increased frost resistance. In particular this applies to plants: which may be cereals such as wheat or maize; or dicotyledons such as soya, tomato or lettuce. Such plants also form part of our invention.

AFPs according to the invention can conveniently be used in several products, preferably in food products which are frozen or intended for freezing. Examples of such food products are: frozen food products such as vegetables, sauces, soups, snacks, dairy products and frozen confectionery, under which term we include sorbet, water-ice, granites, frozen fruit purees and milk-containing frozen confections such as ice-cream, frozen yoghurt or custards, sherbet and ice-milk. Preferred food products are

frozen vegetables and frozen confectionery, e.g. ice-cream, water-ice. If dry-mixes or concentrates are used, the concentration may be higher in order to ensure that the level in the final frozen product is within the desired range. Preferred levels of AFP are from 0.00005 to 0.3%, particularly from 0.0001 to 0.2% by weight of final product.

It is not necessary to add the AFP in highly purified form: it may be added as a composition containing the AFP, for example an extract of the organism that produces the AFP.

Frozen confectionery according to the invention can be produced by any suitable method known to the art. Preferably all ingredients of the formulation are fully mixed together at or above ambient temperature before freezing. The level of solids in the frozen confection (e.g., sugar, fat, flavoring) is suitably adjusted to be at least 3% and normally in the range 10 to 70%, particularly 40 to 70%, by weight of the final product.

Demonstrations of the practical utility of AFP derived from *Marinomonas protea*.

Example 6

Two important requirements for an AFP-producing micro-organism to be of practical utility is that its culturing can be readily scaled-up and that the AFP-activity is stable. For example, susceptibility to denaturation by heat makes it more difficult to use the AFP in foodstuffs such as ice cream that are pasteurised during manufacture.

Culturing *Marinomonas protea* in "shake flasks" (9 litres scale)

Isolate 196 (*Marinomonas protea*) was cultured in 18 individual 1 L Erlenmeyer flasks containing 500 ml of sterile Sea Salts Broth (sea salts (Sigma) 40g/L; peptone (Fisher) 5g/L; yeast extract (Difco) 2g/L). Flasks were inoculated aseptically with 100 µl of an existing *M. protea* culture. Cultures were grown statically at 15°C for 7 days in a Sanyo incubator. The cultures showed signs of turbidity following 2 days at 15°C. On day 7 the incubator temperature was adjusted to 4°C and maintained at this temperature for a further 7 days to induce antifreeze protein (AFP) production.

Following 7 days cold induction at 4 °C, cells from the 9 L of culture were collected in 6 sterile 300 ml containers by repeated centrifugation. Centrifugation was carried out at 7000 rpm for 20 minutes and at 4 °C using a Sorvall F-16/250 fixed angle rotor in a Sorvall Instruments RC5C centrifuge. Following each centrifugation step the supernatant was discarded and further culture added and centrifugation repeated. In this way, pellets were accumulated in the 6 containers until the 9 L of culture was harvested. The six containers and associated pellets were stored at -80°C until required for protein extraction and SPLAT testing.

Protein extraction from 9 litre culture and splat testing for AFP activity

Pellets were washed by gentle resuspension in 10 ml of ice cold 10 mM Tris/HCl (pH 7.0). A 1 ml aliquot of the resuspended cells was centrifuged in an Eppendorf tube for 5 minutes at 13,000 rpm in a microcentrifuge (MSE Micro Centaur). The 'wash supernatant' was retained for testing for AFP activity by the SPLAT assay. The pelleted cells (~ 0.2 g) was resuspended in 800 µl of ice cold extraction buffer (50 mM Tris/HCl, pH 7.0) and 200 µl of Bacterial protease inhibitor cocktail supplied by Sigma. [This cocktail contains a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic and metallo-proteases, and aminopeptidases. It comprises 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin-A, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (E-64), bestatin, and sodium EDTA]. The samples were vortexed and then placed in ice, and this process was repeated a total of 8 times. The samples were then centrifuged and the 'extraction supernatant' retained for AFP testing by the SPLAT assay.

SPLAT activity

Material	**SPLAT score**
'Wash supernatant'	**++++**
'Extraction supernatant'	**+++**

Example 7

Induction of AFP by cold-shocking a culture at 8°C

For the AFP production to be readily scaled-up, it is important that cold-shocking can be effective at a range of different temperatures. For example, it is difficult to operate fermenters at temperatures below 8°C. Therefore, to be able to scale-up production in fermenters, it must be possible to induce expression of AFP by cold-shocking at temperatures of 8°C or above. This example shows that *Marinomonas protea* cultures can be induced to express AFP at 8°C.

Isolate 196 (*Marinomonas protea*) was cultured in a 1 L Erlenmeyer flask containing 500 ml of sterile Sea Salts Broth (sea salts (Sigma) 40g/L; peptone (Fisher) 5g/L; yeast extract (Difco) 2g/L). The flask was inoculated aseptically with 100 µl of an existing *M.protea* culture. The culture was grown statically at 15°C for 7 days in a Sanyo incubator. The culture showed signs of turbidity following 2 days at 15°C. On day 7, the culture was transferred to a Conviron incubator set to 8°C and this temperature was maintained for a further 7 days to induce antifreeze protein (AFP) production.

Following 7 days cold induction at 8 °C, the *M. protea* culture was harvested and tested for AFP activity by the SPLAT assay. The 500ml of culture was collected in a 50ml container by repeated centrifugation. Centrifugation was carried out at 9000 rpm for 5 minutes at 4°C using a Sorvall SS34 rotor in a Sorvall Instrument RC5C centrifuge.

Following each centrifugation step, the supernatant was discarded and further culture added and centrifugation repeated. In this way a pellet accumulated in the container until the 500ml of culture was harvested. The resulting cell pellet weighed 0.9g.

The 0.9g of pelleted cells were gently resuspended in 1ml of ice cold extraction buffer (25 mM Tris/HCl, pH 7.0) and 0.5ml of Bacterial protease inhibitor cocktail supplied by Sigma (see Example 6). The sample was vortexed and then placed on ice, and this process was repeated a total of 8 times. The sample was then centrifuged for 10 minutes at 18000rpm using a Sorvall SS34 rotor in a Sorvall Instrument RC5C centrifuge and the 'extraction supernatant' was filtered using a 0.2 μm filter and retained for SPLAT activity determination (see table).

Material	SPLAT score
'Extraction supernatant'	++++(+)

Example 8

Heat stability of AFP extracted from *Marinomonas protea*

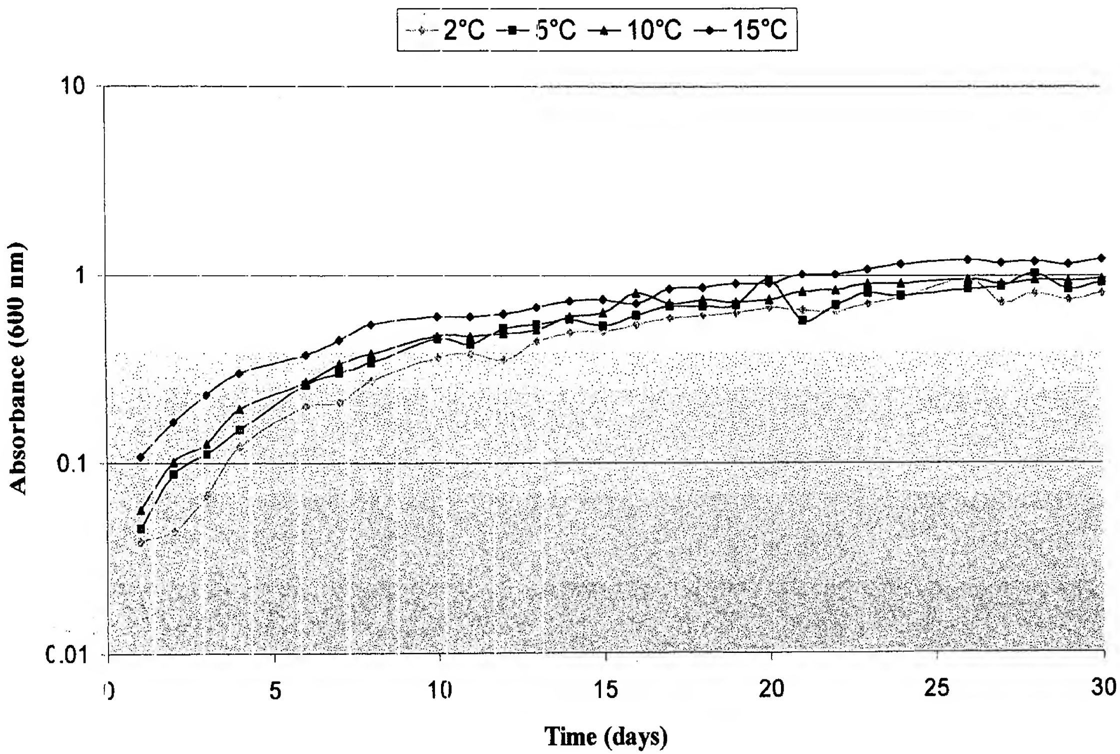
An AFP-containing extract was prepared from a culture of *Marinomonas protea*, essentially as described in Example 6. 25μl aliquots (5 in total) were placed in 500μl Eppendorf tubes and tested for heat stability. Four of the tubes were placed in a bath of boiling water. Each tube was exposed to the heat treatment for a different length of time: either 1,

2, 5, or 15 minutes. The fifth tube was used as a control and was not exposed to the heat treatment. After the heat treatment, all tubes were immediately placed on ice and then assayed for AFP activity using the splat assay.

SPLAT activity following boiling

Treatment	**SPLAT score**
Marinomonas protea *extract, not boiled*	+++++
Marinomonas protea *extract, boiled 1min*	+++++
Marinomonas protea *extract, boiled 2min*	+++++
Marinomonas protea *extract, boiled 5min*	+++++
Marinomonas protea *extract, boiled 15min*	++++

As the AFP extracted from *Marinomonas protea* can survive boiling it should be suitable for use in food-stuffs that are pasteurised during their manufacture. For example, an ice-cream "mix" would typically be pasteurised by holding at a temperature of 82°C for 25 seconds before cooling and freezing. This example shows that the AFP extracted from *Marinomonas protea* could undergo such a pasteurisation process without loss of AFP activity.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10°C
15°C
10
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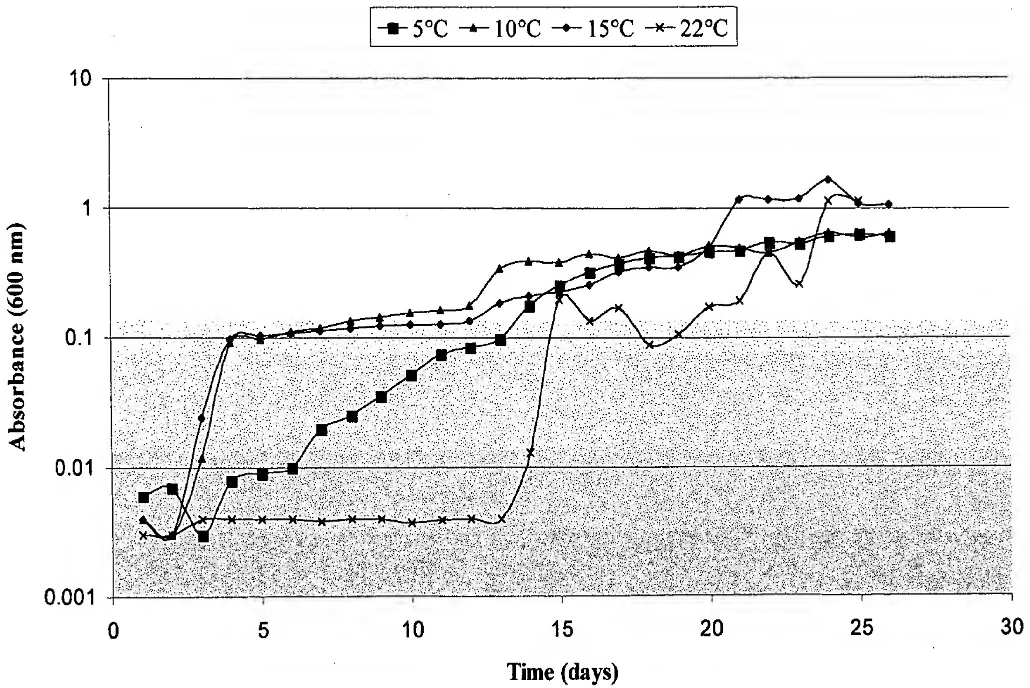
0.01
0
5
10
15
20
25
30
Absorbance (600 nm)
Time (days)

Figure 1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10°C
15°C
22°C
10
1
0.1
0.01
0.001
Absorbance (600 nm)
0
5
10
15
20
25
30
Time (days)

Figure 2

Figure 3

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Figure 4.

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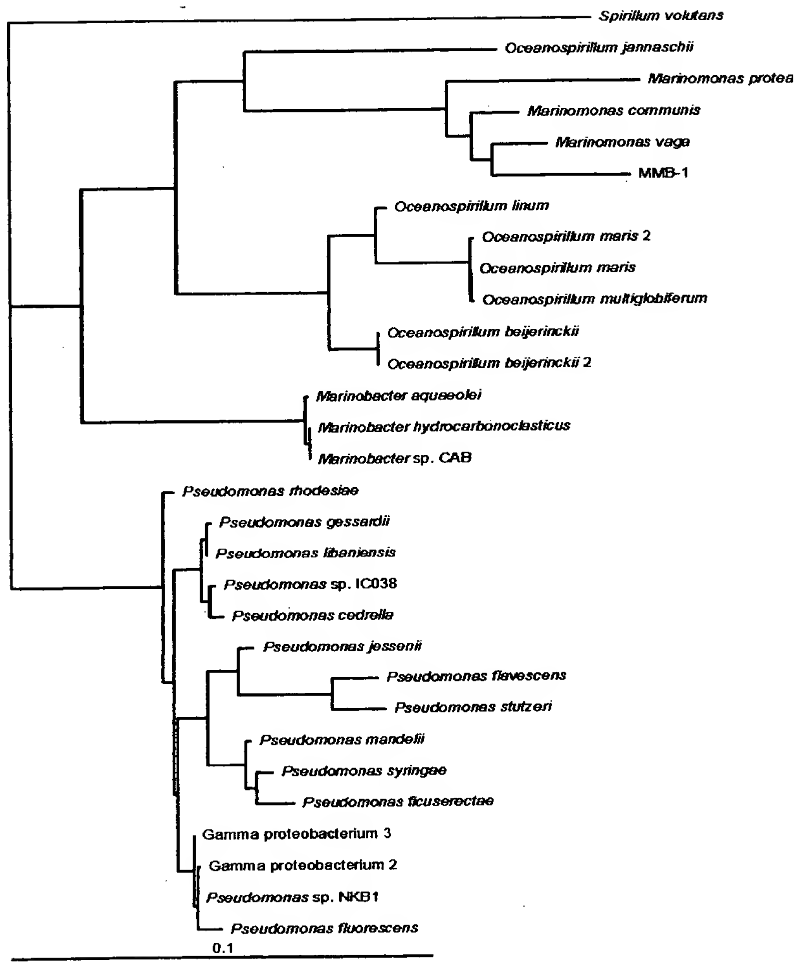
Figure 4 (cont)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Marinobacter hydrocarbonoclasticus
Marinobacter sp. CAB
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Figure 5.